

system employs a phage  $\lambda$  P<sub>L</sub> promoter and a cl857ts thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection ("ATCC"), which incorporate derivatives of the PL promoter, include plasmid pHUB2 (resident in *E. coli* strain JMB9 (ATCC 37092)) and pPLc28 (resident in *E. coli* RR1 (ATCC 53082)).

[084] DNA encoding one or more of the polypeptides of the invention may be cloned in-frame into the multiple cloning site of an ordinary bacterial expression vector. Ideally the vector would contain an inducible promoter upstream of the cloning site, such that addition of an inducer leads to high-level production of the recombinant protein at a time of the investigator's choosing. For some proteins, expression levels may be boosted by incorporation of codons encoding a fusion partner (such as hexahistidine) between the promoter and the gene of interest. The resulting "expression plasmid" may be propagated in a variety of strains of *E. coli*.

[085] For expression of the recombinant protein, the bacterial cells are propagated in growth medium until reaching a pre-determined optical density. Expression of the recombinant protein is then induced, e.g., by addition of IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside), which activates expression of proteins from plasmids containing a lac operator/promoter. After induction (typically for 1-4 hours), the cells are harvested by pelleting in a centrifuge, e.g., at 5,000 x G for 20 minutes at 4°C.

[086] For recovery of the expressed protein, the pelleted cells may be resuspended in ten volumes of 50 mM Tris-HCl (pH 8)/1 M NaCl and then passed two or three times through a French press. Most highly expressed recombinant proteins form insoluble aggregates known as inclusion bodies. Inclusion bodies can be purified

away from the soluble proteins by pelleting in a centrifuge at 5,000 x G for 20 minutes, 4°C. The inclusion body pellet is washed with 50 mM Tris-HCl (pH 8)/1% Triton X-100 and then dissolved in 50 mM Tris-HCl (pH 8)/8 M urea/0.1 M DTT. Any material that cannot be dissolved in 50 mM Tris-HCl (pH 8)/8 M urea/0.1 M DTT may be removed by centrifugation (10,000 x G for 20 minutes, 20°C). The protein of interest will, in most cases, be the most abundant protein in the resulting clarified supernatant. This protein may be "refolded" into the active conformation by dialysis against 50 mM Tris-HCl (pH 8)/5 mM CaCl<sub>2</sub> /5 mM Zn(OAc)<sub>2</sub>/1 mM GSSG/0.1 mM GSH. After refolding, purification can be carried out by a variety of chromatographic methods such as ion exchange or gel filtration. In some protocols, initial purification may be carried out before refolding. As an example, hexahistidine-tagged fusion proteins may be partially purified on immobilized Nickel.

[087] While the preceding purification and refolding procedure assumes that the protein is best recovered from inclusion bodies, those skilled in the art of protein purification will appreciate that many recombinant proteins are best purified out of the soluble fraction of cell lysates. In these cases, refolding is often not required, and purification by standard chromatographic methods can be carried out directly.

#### Yeast Expression Systems

[088] Polypeptides of the invention can also be expressed in yeast host cells, preferably from the *Saccharomyces* genus (e.g., *S. cerevisiae*). Other genera of yeast, such as *Pichia* or *Kluyveromyces* (e.g. *K. lactis* ), can also be employed. Yeast vectors will often contain an origin of replication sequence from a 2μ yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for

polyadenylation, sequences for transcription termination, and a selectable marker gene. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothioneine, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255:2073, 1980), or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* 7:149, 1968; and Holland et al., *Biochem.* 17:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EPA-73,657 or in Fleer et. al., *Gene*, 107:285-195 (1991); and van den Berg et. al., *Bio/Technology*, 8:135-139 (1990). Another alternative is the glucose-repressible ADH2 promoter described by Russell et al. (*J. Biol. Chem.* 258:2674, 1982) and Beier et al. (*Nature* 300:724, 1982). Shuttle vectors replicable in both yeast and *E. coli* can be constructed by inserting DNA sequences from pBR322 for selection and replication in *E. coli* (Amp<sup>r</sup> gene and origin of replication) into the above-described yeast vectors.

[089] The yeast  $\alpha$ -factor leader sequence can be employed to direct secretion of one or more of the disclosed polypeptides. The  $\alpha$ -factor leader sequence is often inserted between the promoter sequence and the structural gene sequence. See, e.g., Kurjan et al., *Cell* 30:933, 1982; Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, 1984; U.S. Patent 4,546,082; and EP 324,274. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence can be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.